Ability of different types and doses of tannin extracts to modulate *in vitro* ruminal biohydrogenation in sheep

D. Carreño, G. Hervás, P.G. Toral, A. Belenguer, P. Frutos *

Instituto de Ganadería de Montaña (CSIC-ULE), Finca Marzanas s/n, 24346 Grulleros, León, Spain

* Corresponding author. Tel.: +34 987 317 156; fax: +34 987 317 161.

E-mail address: p.frutos@csic.es (P. Frutos).

ABSTRACT

The ability of tannins to interfere with ruminal biohydrogenation (BH) and modulate the fatty acid (FA) profile of ruminant-derived products is highly controversial, which is probably related to the type of tannin and the dosage rate. Therefore, this *in vitro* study was conducted to analyse the effect of 4 commercial extracts of tannins (from chestnut, oak, quebracho and grape) at 4 doses (20, 40, 60 and 80 g/kg diet DM) with the aim of selecting an effective treatment to modulate the BH of unsaturated FA. Two *in vitro* assays with batch cultures of rumen microorganisms, using cannulated ewes as donors of rumen inocula, were performed. The incubated substrate (a total mixed ration similar to that fed to the animals) was supplemented with 20 g of sunflower oil/kg DM. The first experiment followed a 4 × 4 + 1 design (*i.e.*, 4 types of tannins × 4 doses of each one, and a control), and treatment effects on the FA composition of the ruminal digesta were examined by gas chromatography. On the basis of these results, the second experiment was conducted to make sure that the selected dose and type of tannin would not impair rumen fermentation. To this end, gas production kinetic parameters, extent of degradation, *in vitro* true substrate digestibility, pH, and ammonia and volatile FA concentrations, as well as the bacterial community (by terminal restriction fragment length polymorphism, T-RFLP) were examined. All tannin extracts were able to modulate the *in vitro* BH of unsaturated FA. However, the high dose required in many cases suggests that their efficacy would be rather limited in terms of animal feeding. On the other hand, the oak tannin extract, at a dose of 20 g/kg diet DM, increased total polyunsaturated FA, 18:3n-3, 18:2n-6 and *trans*-11 18:1, and decreased *trans*-10 18:1 and 18:0 rumen concentrations without eliciting any negative response in ruminal fermentation. Although this treatment had no discernible effects on the bacterial community structure and

diversity, a few fragments compatible with uncultured *Lachnospiraceae* species were affected.

Keywords: ewe, fatty acid, condensed tannin, hydrolysable tannin, rumen microbiota, ruminal fermentation

Abbreviations: *A*, cumulative gas production; ADF, acid detergent fibre; AFR, average fermentation rate; aNDF, neutral detergent fibre; BCFA, branched-chain fatty acids; BH, biohydrogenation; *c*, fractional fermentation rate; CHE, chestnut; CLA, conjugated linoleic acid; CP, crude protein; DM, dry matter; DMD, dry matter disappearance; ED, extent of degradation in the rumen; FA, fatty acid; FAME, fatty acid methyl esters; GRA, grape; *iv*TSD, *in vitro* true substrate digestibility; OBCFA, odd- and branched-chain fatty acids; OM, organic matter; PUFA, polyunsaturated fatty acids; QUE, quebracho; TMR, total mixed ration; T-RFLP, terminal restriction fragment length polymorphism; VFA, volatile fatty acid.

1. Introduction

Due to consumers' concerns about food of animal origin and demands for healthier food, considerable effort is being made by ruminant nutrition researchers to develop products that are safe and potentially health-promoting. Such properties have been assigned to unsaturated fatty acids (FA) and particularly to conjugated linoleic acids (CLA) formed in the rumen by microbial biohydrogenation (BH) of certain polyunsaturated FA (PUFA; Lock and Bauman., 2004; Shingfield et al., 2008).

Increased levels of desirable FA in ruminant derived products can be achieved through feeding strategies reducing the extent of BH or facilitating a higher rumen output of *cis*-9 *trans*-11 CLA, the main health-promoting CLA isomer, and especially *trans*-11 18:1, which will act as a precursor of the former in the animal's own tissues (Lock and Bauman, 2004; Shingfield et al., 2008).

Tannins are plant secondary compounds with antibacterial and rumen modulating properties that are able to interfere with BH (McSweeney et al., 2001; Mueller-Harvey, 2006; Vasta et al., 2009a). Some *in vitro* studies have suggested that diet supplementation with these phenolic compounds may be an efficient tool to favourably modify the BH of dietary PUFA and enhance the accumulation of *trans*-11 18:1 due to an inhibition of the last step of BH (Khiaosa-ard et al., 2009; Buccioni et al., 2011). However, some others have reported a general inhibition of BH rather than a specific inhibition of the conversion of *trans*-11 18:1 to 18:0 (Kronberg et al., 2007; Minieri et al., 2014). In any case, this beneficial effect has rarely been validated *in vivo* (Vasta et al., 2009b; Khiaosa-ard et al., 2011) and many experiments seem to point to a different direction (*e.g.*, Benchaar and Chouinard, 2009; Cabiddu et al., 2009).

Results from our studies with lactating ewes fed diets supplemented with a combination of tannin extracts of quebracho and chestnut (10 g/kg diet DM; Toral et al., 2011) or just quebracho (20 g/kg diet DM; Toral et al., 2013) showed that tannin addition was not able to modify milk FA composition towards a potentially healthier profile, especially in the long-term. Recently, Buccioni et al. (2015), using the same tannin extracts (*i.e.*, quebracho and chestnut) but at higher levels (53 g/kg diet DM), observed a slight increase in the milk concentration of linoleic, vaccenic and rumenic acids and a decrease in stearic and saturated FA.

Given the great variation in the structural features and reactivity of different tannins (Álvarez del Pino et al., 2005; Mueller-Harvey, 2006), all these inconsistent results may be attributable to the type and/or concentration of tannins. Thus, there is a void of knowledge about which of the many types of tannins might be potentially more useful for a particular purpose, and also a need for further studies.

Therefore, this *in vitro* study was conducted to analyse the effect of different types and doses of tannins with the aim of selecting an effective treatment to modulate the ruminal BH of unsaturated FA. Once a type of tannin at a practical dose (in terms of cost and avoidance of toxicity; Makkar, 2003) was chosen, a secondary aim, before recommending its test *in vivo*, was to make sure that it would not impair ruminal fermentation.

2. Materials and methods

An *in vitro* trial (Experiment 1) was conducted in batch cultures to assess the effects of different concentrations of a range of tannins on rumen BH. On the basis of the results obtained in this assay, a new *in vitro* trial (Experiment 2) was conducted to test the effect of a selected type and dose of tannin on rumen fermentation and

bacterial community. All experimental procedures were approved and completed in accordance with the Spanish Royal Decree 53/2013 for the protection of animals used for experimental purposes.

2.1. Animals, diet and tannins

In vitro incubations were conducted as outlined previously (Frutos et al., 2004) with rumen fluid collected from 5 ruminally cannulated (40 mm internal diameter) Merino sheep (body weight = 63.6±6.42). All the animals were offered a total mixed ration (TMR, forage:concentrate ratio 50:50), based on alfalfa hay (particle size >4 cm) and concentrates, in two meals (60% at 9:00 h and 40% at 17:00 h) at approximately 0.8 times the voluntary feed intake previously determined *ad libitum* (37 g DM/kg metabolic weight and day). Formulation and chemical composition of the diet is shown in Table 1. Animals had continuous access to clean drinking water.

Four types of commercial oenological tannin extracts (Agrovin S.A., Alcázar de San Juan, Spain) were tested: 2 condensed [quebracho (QUE; *Schinopsis lorentzii* – TanicolMOX) and grape (GRA; *Vitis vinifera* – Tanicol VMax)] and 2 hydrolysable [chestnut (CHE; *Castanea sativa* – Vinitanon) and oak (OAK; *Quercus robur* and *Q. petraea* – Robletan FST)] tannins.

2.2. In vitro experiments

In each experiment, and after an adaptation period of 15 days, rumen fluid inocula (collected in three different days) were obtained via the cannula before the morning feeding. The inocula were immediately taken in thermal flasks to the laboratory where they were strained through a nylon membrane (400 µm; Fisher Scientific S.L., Madrid, Spain) while bubbled with CO_2.

2.2.1. Experiment 1

This trial was conducted using batch cultures of rumen microorganisms (in 16 mL Hungate tubes), following a 4 × 4 + 1 (control) design. Treatments were: 4 types of tannins (quebracho, grape, chestnut and oak) × 4 doses of each one (20, 40, 60 and 80 g/kg DM), and a control.

The incubated substrate was a TMR, similar to that used to feed the animals, supplemented with 20 g of sunflower oil/kg diet DM [Carrefour S.A., Madrid, Spain; containing (g/kg total FA): 16:0 (54.8), 18:0 (44.2), *cis*-9 18:1 (364) and 18:2n-6 (503)]. Both the oil and the tannins were dissolved, respectively, in ethanol 96% and in water at about 30ºC, and added into the tubes just before the incubation started.

Each Hungate tube contained 78 mg DM of the substrate (ground using a hammer-mill fitted with a 0.5 mm screen) that were incubated with 12 mL of a mix (1:2) of strained rumen fluid and phosphate-bicarbonate buffer (Goering and Van Soest, 1970). The pH was adjusted to 6.8 with orthophosphoric acid in order to better simulate ruminal conditions in animals fed a 50:50 forage:concentrate diet.

Tubes were incubated under anaerobic conditions for 12 h (when, according to previous preliminary assays, effects were better detected) in an incubator set at 39.5ºC, and were individually agitated every 6 h. The reaction was stopped by placing the tubes into ice-water for approximately 5-10 min. They were then stored at −80ºC until FA analysis.

2.2.2. Experiment 2

Once the oak tannin extract at a dose of 20 g/kg DM was selected, a new *in vitro* trial was conducted in 125 mL sealed serum flasks to test the effect of this treatment on rumen fermentation and bacterial community.

For each of the three runs, two samples of each treatment (*i.e.*, control and OAK20; 325 mg DM milled to pass a 1 mm screen) and two blanks (*i.e.*, buffered

rumen fluid without substrate) were incubated at 39.5ºC with 50 mL of a mix (1:2) of strained rumen fluid and phosphate-bicarbonate buffer. The pH of the buffer solution was adjusted to 6.8 as in the Experiment 1.

The rate and extent of gas production were determined by measuring head-space gas pressure at 2, 4, 6, 9, 12, 16, 20, 24, 30, 36, 48, 60 and 72 h post-inoculation. Pressure values, corrected for the quantity of organic matter (OM) incubated and gas released from blanks, were used to generate gas volume estimates using a predictive equation, as reported in Frutos et al. (2004). Dry matter disappearance (DMD; g/kg) after 72 h incubation was estimated by filtering residues using pre-weighed sintered glass crucibles (100–160 µm; Pyrex, Stone, UK).

In addition, three more flasks per treatment and run were incubated for 24 h. Once the reaction was stopped, the pH was measured in two flasks and centrifuged samples (at 976 × *g* for 10 min) were collected for ammonia and volatile fatty acid (VFA) analysis. Values of DMD and *in vitro* true substrate digestibility (*iv*TSD) were estimated by filtering the residues using pre-weighed sintered glass crucibles (100–160 µm; Pyrex, Stone, UK) and determining the neutral detergent fibre content, as reported in Frutos et al. (2004). The third flask was immediately frozen at –80ºC for subsequent microbial DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analysis.

2.3. Chemical analysis

Feed samples were prepared (ISO 6498:2012) and analysed for DM (ISO 6496:1999), ash (ISO 5984:2002), and crude protein (ISO 5983-2:2009). Neutral and acid detergent fibres (aNDF and ADF) were determined using an Ankom^{2000} fibre analyser (Ankom Technology Methods 13 and 12, respectively; Ankom Technology Corp., Macedon, NY, USA, https://ankom.com/procedures.aspx); the former was

assayed with sodium sulfite and α-amylase, and both were expressed with residual ash. The content of ether extract in the diets was determined by the Ankom Filter Bag Technology (Ankom Technology Method 2; Ankom Technology Corp.). Fatty acid methyl esters (FAME) of lipid in freeze-dried samples of TMR and in the sunflower oil were prepared in a one-step extraction-transesterification procedure, as outlined previously by Shingfield et al. (2003).

Rumen samples for FA composition were freeze-dried directly in the Hungate tubes. The lipids were then extracted using a mixture of hexane and isopropanol (3:2, vol/vol) and converted to FAME by sequential base-acid catalysed transesterification (Toral et al., 2010). Methyl esters were separated and quantified using a gas chromatograph (Agilent 7890A GC System, Santa Clara, CA, USA) equipped with a flame-ionisation detector and a 100-m fused silica capillary column (0.25 mm i.d., 0.2-μm film thickness; CP-SIL 88, CP7489, Varian Ibérica S.A., Madrid, Spain) and hydrogen as the carrier gas. Total FAME profile in a 2 μL sample volume at a split ratio of 1:50 was determined using a temperature gradient programme (Shingfield et al., 2003). Isomers of 18:1 were further resolved in a separate analysis under isothermal conditions at 170ºC (Shingfield et al., 2003). Peaks were identified based on retention time comparisons with commercially available authentic standards (Nu-Chek Prep., Elysian, MN, USA; Sigma-Aldrich, Madrid, Spain; and Larodan Fine Chemicals AB, Malmö, Sweden; Toral et al., 2010), cross referencing with chromatograms reported in the literature (Shingfield et al., 2003; Toral et al., 2010) and comparison with reference samples for which the FA composition was determined based on gas chromatography analysis of FAME and gas chromatography-mass spectrometry analysis of corresponding 4,4-dimethyloxazoline derivatives (Toral et al., 2010).

The ammonia concentration was determined by colorimetry, and VFA by gas chromatography, using crotonic acid as the internal standard, both in centrifuged samples (Frutos et al., 2004).

2.4. DNA extraction and T-RFLP analysis

Frozen samples were freeze-dried and thoroughly mixed before DNA extraction, which was conducted in duplicate using the Qiagen QIAmp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA, USA), with the modification of a greater temperature (95ºC) to improve cell lysis. Duplicate samples were combined and used as templates for T-RFLP analysis of bacterial 16S rRNA genes, which were performed as described previously (Castro-Carrera et al., 2014), using three restriction enzymes (*HhaI*, *MspI* and *HaeIII*). Determination of the sizes of the terminal restriction fragments (T-RF) was completed with the size standard ET 900-R (GE Healthcare Life Sciences, Buckinghamshire, UK) and the GeneMarker Analysis software (SoftGenetics, State College, PA, USA). In order to infer the bacterial composition of the samples, *in silico* restriction for the major rumen bacteria with the primers and the enzymes used were obtained from the Ribosomal Database Project II website (http://rdp.cme.msu.edu/index.jsp; Cole et al., 2014).

2.5. Calculations and statistical analysis

Gas production data were fitted to an exponential model using SAS software package (version 9.3, SAS Institute Inc., Cary, NC, USA) to provide parameters describing gas release in terms of cumulative gas production (*A*, mL of gas/g of OM incubated) and fractional fermentation rate (*c*, /h). Average fermentation rate (AFR, mL of gas/h) and extent of degradation in the rumen (ED, g/kg of DM) were estimated assuming a rumen particulate outflow (k_p, /h) of 0.042, according to the

following equations: ED = (c × DMD)/(c + k_p), and AFR = (A × c)/(2 × ln2), where DMD = *in vitro* DM disappearance after 72 h of incubation.

Data from T-RFLP (size, bp, and peak area for each T-RF) were analysed for peak filtering and binning as outlined by Abdo et al. (2006), and used to determine the relative abundance of each fragment over the total peak area, as well as the diversity indices (number of T-RF or richness, and Shannon index; Hill et al., 2003). Multivariate analysis of variance (MANOVA) of the relative abundance data of each T-RF was conducted, using the R-project software (www.r-project.org, version 3.1.1), to assess the effect of experimental treatment on the whole bacterial structure.

FA composition of ruminal digesta from the Experiment 1 was analysed by a one-way ANOVA using the MIXED procedure of SAS with a model that included the fixed effect of experimental treatment. Run nested within treatment was designated as the random effect. Orthogonal polynomial contrasts were used to evaluate linear (L), quadratic (Q) and cubic (C) components of the response to incremental amounts of each tannin extract. Rumen fermentation data from the Experiment 2 were analysed using the MIXED procedure of SAS with a model that included the fixed effect of experimental treatment and the random effect of run nested within the treatment. Relative abundances of each T-RF were analysed similarly, although some data did not satisfy the assumptions of normality and were transformed to $\log_{10}$ (n + c) ("c" being a constant of the same order of magnitude as the variable). Differences were declared significant at $P<0.05$ and considered a trend towards significance at $P<0.10$. Means were separated through the "pdiff" option of the "lsmeans" statement of the MIXED procedure, and least square means are reported.

3. Results

3.1. FA composition of ruminal digesta (Experiment 1)

As shown in Table 2 and Figure 1, all tannins and doses, with the exception of QUE80, proved to be able to slightly reduce the concentration of 18:0 ($P<0.001$) after 12 h incubations. Low and moderate doses increased concentrations of 18:2n-6 and 18:3n-3 (up to 90 and 86%, respectively; $P<0.01$). However, only some doses of 60 and 80 g/kg tended to augment *cis*-9 18:1. Likewise, concentrations of *cis*-9 *trans*-11 18:2 were only favoured by 60 g/kg of QUE and CHE, and 80 of GRA and OAK ($P<0.001$), the highest value representing an increase of 128% compared to the control. No significant effect of the tannin treatment was observed on the digesta concentration of *trans*-9 *cis*-12 18:2.

A tendency to a greater accumulation of *trans*-11 18:1 was only detected with 60 and 80 g/kg of GRA and CHE, and 20 of OAK (up to 16%; $P<0.10$). The concentration of *trans*-10 18:1 showed an irregular behaviour: although QUE60 increased it, most other treatments (*e.g.*, QUE, CHE and OAK at 20 g/kg) decreased it ($P<0.001$).

These variations were accompanied by a number of changes in several odd and branched-chain FA and also in some oxo-FA concentrations (see Table 3 and Figure 1). Thus, the proportion of 14:0 *iso* was increased by QUE and CHE at 20, 40 and 60 g/kg and by OAK at 20 and 40 g/kg ($P<0.01$). No differences were found in 15:0 *iso* ($P>0.10$) while many treatments increased 15:0 *anteiso*, and 15:0 was higher in QUE40 and CHE20 and 40 ($P<0.05$). All tannins decreased 17:0 when added at doses of 20, 40 and 60 g/kg, and GRA and CHE also at 80 g/kg. Overall, despite the erratic pattern, all tannin extracts led to general increases in both branched-chain (BCFA) and odd- and branched-chain (OBCFA) fatty acids ($P<0.01$).

3.2. Rumen fermentation and bacterial community (Experiment 2)

As shown in Table 4, addition of OAK20 had no effect on any of the rumen fermentation characteristics that were analysed (*e.g.*, gas production kinetic parameters, extent of degradation, pH, ammonia and VFA concentrations, molar proportions of VFA, etc.; $P>0.10$).

Regarding the microbiota, MANOVA revealed that OAK20 had no discernible effects on the bacterial community structure ($P>0.10$). Neither did the diversity indices (richness and Shannon) differ between treatments in data derived from the enzymes *HhaI*, *MspI* and *HaeIII* ($P>0.10$; Table 5). However, the OAK20 induced variations in the relative abundances of a few T-RF (Table 5), such as an increase in some fragments that may correspond to uncultured bacteria of the class *Clostridia* (750 bp with *HhaI*; $P<0.10$) or the family *Lachnospiraceae* (65 bp with *HhaI*, 293 bp with *MspI*, and 277 bp with *HaeIII*; $P<0.05$).

4. Discussion

Due to its major influence on the FA composition of ruminant meat and milk, a great deal of effort has been directed towards modulation of lipid metabolism in the rumen (Lock and Bauman, 2004; Shingfield et al., 2008). The ability of tannins to contribute to this goal is highly controversial, which is most probably related to their type and dosage rate, and highlights the need to further investigate on this issue.

As shown in Figure 1, although most FA concentrations follow a similar pattern of response to tannin extracts (*e.g.*, decreases in 17:0 and 18:0 or increases in 18:2n-6 and 18:3n-3), there were also many exceptions in their effects (*e.g.*, on *trans*-10 and *trans*-11 18:1 or on *cis*-9 *trans*-11 18:2) due to both the type of tannin and the dose.

Starting from the type, there are some studies on the use of commercial extracts of quebracho, and also of chestnut tannins (*e.g.*, Vasta et al., 2009a; Toral et al., 2011; Buccioni et al., 2015), although their results are rather inconsistent. Nevertheless, and despite some works have been conducted with grape seeds (*e.g.*, Correddu et al., 2015), reports are very limited for marketable extracts of grape or oak tannins.

Tannins comprise a very wide and heterogeneous group of phenolic compounds with different chemical and structural features (Mueller-Harvey, 2006). Hence, dissimilarities in procyanidin/prodelphinidin ratios, degree of galloylation, molecular weights, etc. would account for major variations in their ability to bind to other molecules or to affect microorganisms, and consequently in their effects, in this case on ruminal BH. In line with this, for example, the inclusion of an extract of condensed tannins from *Acacia mearnsii* (79 g/kg DM) inhibited the *in vitro* conversion of *trans*-11 18:1 to 18:0, while the same amount of tannins from *Onobrychis viciifolia* decreased the hydrogenation of linoleic and linolenic acids but had no effect on the last step of BH (Khiaosa-ard et al., 2009).

Regarding the key issue of the dosage rate, we chose 4 doses to have a wide range that allow us to detect effects that may help to understand the underlying mechanisms of tannins. However, only the low (20 g/kg) and perhaps the moderate (40 g/kg) concentrations might be of interest in terms of animal feeding. Even if the 60 and 80 g/kg doses had shown promising effects on the BH process, they could be not only detrimental to animal performance but also impractical under farm conditions due to their cost. This occurs, for instance, in some *in vitro* assays that found a beneficial impact of tannins on BH but at very high levels (up to 160 g/kg DM; Vasta et al., 2009a). The difficulty in selecting dosage rates of plant secondary

compounds to positively affect a particular parameter without conferring a negative response in others has been previously reported in several occasions (*e.g.*, Benchaar et al., 2008; Toral et al., 2013).

In any case, regardless of the practicality, our results do not clearly point to a greater ability of a particular dose to modulate the BH process towards a potentially healthier FA profile, suggesting interactions between types of tannins, doses and specific steps of the BH pathways. Thus, for example, only doses of 60 and 80 g/kg were able to promote the accumulation of *cis*-9 *trans*-11 CLA, while those of 20, 40 and 60 g/kg increased the concentration of total PUFA, linoleic and linolenic acids, and QUE80 was the only treatment unable to decrease the proportion of 18:0. In agreement with this, Buccioni et al. (2011) observed that the effect of tannins on the BH process was, in many cases, stronger with a lower dose (49 vs. 82 g/kg DM).

Overall, our results are consistent with those of other *in vitro* studies (*e.g.*, Kronberg et al., 2007; Minieri et al., 2014) and suggest a general inhibition of the BH rather than the specific negative effect on the conversion of *trans*-11 18:1 to 18:0 that had been detected in some *in vitro* assays (Khiaosa-ard et al., 2009, Vasta et al., 2009a, Buccioni et al., 2011). However, as mentioned in the introduction, they are in disagreement with the slight but positive effects reported by Buccioni et al. (2015) in ewes fed quebracho and chestnut tannin extracts at a dose of 53 g/kg diet DM. Although differences between *in vitro* and *in vivo* results cannot be ruled out, after a comprehensive comparison of these studies, the reason for the discrepancy is still uncertain. It is noteworthy, however, that in this latter work, tannin extracts replaced bentonite from the diet, which might partly explain some differences with the control diet, due to the potential effects of this clay on BH (Jeronimo et al., 2010).

Despite all tannin extracts tended to favour a slight accumulation of *trans*-11 18:1 (with a $P<0.10$ only with doses of 60 and 80 g/kg of grape and chestnut, and 20 of oak), their effect on the concentration of *trans*-10 18:1, a FA with an uncertain involvement in consumers' health and animals' performance (Shingfield et al., 2008), was highly variable. This is in line with inconsistent results found in the literature (*e.g.*, Cabiddu et al., 2009, Abbedou et al., 2011; Toral et al., 2011). Nevertheless, the formation of *trans*-10 18:1 was not promoted at the expense of *trans*-11 18:1, which may be related to the basal diet (Vasta et al., 2009b) and would indicate only small deviations from major ruminal BH pathways.

In this regard, concentrations of *trans*-9 *cis*-12 18:2 were investigated to test the hypothesis that tannins would benefit microbial populations able to metabolise *cis*-9 *cis*-12 18:2 via mechanisms other than isomerisation of the *cis*-12 double bond. However, this minor BH pathway (Honkanen et al., 2012), observed in sheep fed quebracho tannins (Toral et al., 2013), was not evident in our current assay.

Different effects of the extracts on the BH of different unsaturated FA would suggest a distinct sensitivity to tannins of the microorganisms involved in each step of the process (Buccioni et al., 2011) because all changes are supposed to be mediated by the impact of tannins on the microbiota. This was supported by the variations observed in odd- and branched-chain FA, which are known to be largely derived from bacteria (Fievez et al., 2012). The quoted review by Fievez et al. (2012) related an increased proportion of cellulolytic bacteria with a high *iso* FA content, while increased amylolytic populations would facilitate a higher content of *anteiso* and linear odd-chain FA. Yet, the erratic pattern observed in our incubations does not allow us to attribute differences to specific alterations in these two kinds of bacteria. Changes in odd- and branched-chain FA reflecting shifts in rumen microorganisms

are also supported by variations in some keto-FA (*e.g.*, 10-oxo- and 13-oxo-18:0), which suggest alteration of the ruminal pathways (Toral et al., 2010, 2012).

To recapitulate, the treatment showing a more promising behaviour at a practical dose was OAK20, which increased total PUFA, 18:3n-3, 18:2n-6 and *trans*-11 18:1, and decreased *trans*-10 18:1 and 18:0 concentrations. Therefore, the second experiment was conducted to ensure that this oenological extract of hydrolysable tannins would not elicit a negative response in ruminal fermentation.

Although the attribution of more toxic and less efficient results to hydrolysable than to condensed tannins has proved simplistic and erroneous, some generalisations still persist (Mueller-Harvey, 2006). However, the OAK20 modulated BH but did not detrimentally affect any of the rumen fermentation characteristics that were analysed, most likely due to the small amount added to the diet. A number of studies have demonstrated this dose-dependent effect of tannins (*e.g.*, Hervás et al., 2003, Makkar, 2003), which is even applicable to their capacity to reduce rumen ammonia concentration due to their strong inhibitory effect on proteolysis (Makkar, 2003; Frutos et al., 2004; Mueller-Harvey, 2006).

Finally, regarding the microbial analyses, the low dose of tannins precluded major changes and therefore discernible effects were observed neither in the bacterial community structure nor in the diversity indices. Furthermore, the issue of the shifts in specific bacteria involved in BH is rather complicated because recent studies question previous reports indicating that BH is achieved only by a small group of bacteria and suggest that yet uncultivated species would play a relevant role (Huws et al., 2011; Toral et al., 2012, Castro-Carrera et al., 2014).

In this assay, some T-RF affected by the addition of OAK20 are compatible with uncultured species belonging to the family *Lachnospiraceae*, which include

bacterial strains that have been related to lipid metabolism, both *in vitro* (Paillard et al., 2007; Boeckaert et al., 2009) and *in vivo* (Huws et al., 2011; Toral et al., 2012). Concerning the tolerance of the microbes of this diverse group to the presence of tannins, reports in the literature are once again inconsistent. For example, it has been reported that sainfoin (*Onobrychis viciifolia*) tannins inhibit the growth of a strain of *Butyrivibrio fibrisolvens in vitro* (Jones et al., 1994) but tannin-resistant bacteria of this genus have been identified in the rumen (Odenyo et al., 2001). Indeed, Vasta et al. (2010) observed in lambs that the addition of quebracho tannins to the diet increased its abundance.

5. Conclusions

The four oenological tannin extracts (quebracho, grape, chestnut and oak) that were examined in this study seem to be able to modulate the *in vitro* BH of unsaturated FA. However, the high dose required in many cases suggests that their efficacy would be rather limited in terms of animal feeding. On the other hand, the oak tannin extract, at a practical dose of 20 g/kg diet DM, increases total PUFA, 18:3n-3, 18:2n-6 and *trans*-11 18:1, and decreases *trans*-10 18:1 and 18:0 rumen concentrations without eliciting any negative response in ruminal fermentation. Further studies would be now necessary to examine if these positive effects are extended to *in vivo* conditions.

Conflict of interest

No conflict of interest.

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Table 1

Formulation and chemical composition of the experimental diet.

	TMR[1]	SEM[2]
Ingredients, g/kg of fresh matter		
Dehydrated alfalfa hay	500	-
Whole corn grain	140	-
Whole barley grain	100	-
Soybean meal solvent 440 g CP/kg	150	-
Sugar beet pulp, pellets	50	-
Molasses, liquid	40	-
Mineral supplement[3]	18	-
Vitamin supplement[4]	2	-
Chemical composition, g/kg DM		
Organic matter	900	3.15
Crude protein	187	6.08
Neutral detergent fibre	311	24.3
Acid detergent fibre	218	21.1
Ether extract	23.5	1.80

[1]n = 6. Contained (g/kg of total fatty acids): 16:0 (252), 18:0 (53.8), *cis*-9 18:1 (139), 18:2n-6 (346) and 18:3n-3 (119).

[2]SEM=standard error of the mean.

[3]Contained (g/kg): $CaCO_3$ (556), Ca_2HPO_4 (222), and NaCl (222).

[4]*VITAFAC Ovino 0.2% AC* (DSM Nutritional Products S.A., Madrid, Spain). Declared as containing: vitamin A (4,000,000 IU/kg), vitamin D3 (1,000,000 IU/kg), vitamin E (5 g/kg), iron (17.5 g/kg), manganese (20 g/kg), cobalt (50 mg/kg), iodine (250 mg/kg), zinc (15 g/kg), selenium (100 mg/kg), sepiolite (100 g/kg), calcium (26.2 g/kg), and magnesium (6.15 g/kg).

Table 2

Effect of incremental levels of different tannin extracts (quebracho, QUE; grape, GRA; chestnut, CHE; oak, OAK) on 18:0 and unsaturated fatty acid concentration (g/100 g of total fatty acids) after 12-h *in vitro* incubation with rumen inoculum from sheep[1].

	18:0	*cis*-9 18:1	*trans*-10 18:1	*trans*-11 18:1	*cis*-9 *trans*-11 18:2[2]	*trans*-9 *cis*-12 18:2	18:2n-6	18:3n-3	PUFA[3]
Control	61.2^{a}	2.06^{c}	0.387^{b-e}	5.15^{d}	0.088^{d-f}	0.033	0.877^{f}	0.175^{f}	1.38^{e}
QUE20	58.8^{c-e}	2.24^{bc}	0.354^{f-h}	5.35^{cd}	0.092^{d-f}	0.035	1.31^{b-d}	0.278^{a-d}	2.07^{b-d}
QUE40	58.2^{ef}	2.40^{bc}	0.321^{h}	5.57^{a-d}	0.100^{d-f}	0.034	1.41^{a-d}	0.314^{ab}	2.21^{ab}
QUE60	58.7^{de}	2.45^{ab}	0.423^{a}	5.61^{a-d}	0.142^{c}	0.036	1.42^{a-d}	0.286^{a-c}	2.21^{ab}
QUE80	60.4^{ab}	2.31^{bc}	0.406^{a-c}	5.29^{cd}	0.098^{d-f}	0.038	1.11^{c-f}	0.213^{d-f}	1.77^{de}
GRA20	58.4^{ef}	2.21^{bc}	0.398^{a-d}	5.30^{cd}	0.081^{d-f}	0.038	1.31^{b-d}	0.285^{a-c}	2.07^{b-d}
GRA40	58.4^{ef}	2.29^{bc}	0.368^{d-g}	5.49^{a-d}	0.114^{c-e}	0.033	1.29^{b-d}	0.256^{a-e}	2.13^{b}
GRA60	58.1^{ef}	2.48^{ab}	0.393^{a-e}	5.68^{a-c}	0.091^{d-f}	0.042	1.27^{b-e}	0.234^{c-f}	2.21^{ab}
GRA80	58.9^{c-e}	2.60^{a}	0.400^{a-d}	5.95^{a}	0.201^{a}	0.037	1.14^{b-f}	0.230^{c-f}	1.86^{b-d}
CHE20	58.4^{ef}	2.28^{bc}	0.346^{gh}	5.25^{cd}	0.096^{d-f}	0.030	1.47^{ab}	0.307^{ab}	2.25^{ab}
CHE40	58.4^{ef}	2.34^{a-c}	0.354^{f-h}	5.38^{b-d}	0.100^{d-f}	0.033	1.47^{ab}	0.313^{ab}	2.27^{ab}
CHE60	57.4^{f}	2.60^{a}	0.411^{ab}	5.71^{a-c}	0.186^{ab}	0.042	1.67^{a}	0.325^{a}	2.56^{a}
CHE80	59.8^{b-d}	2.37^{ab}	0.386^{b-f}	5.65^{a-c}	0.114^{c-e}	0.043	1.08^{d-f}	0.198^{ef}	1.74^{c-e}
OAK20	58.0^{ef}	2.34^{abc}	0.352^{gh}	5.86^{ab}	0.076^{ef}	0.037	1.47^{a-c}	0.299^{a-c}	2.27^{ab}
OAK40	58.3^{ef}	2.30^{bc}	0.376^{c-g}	5.59^{a-d}	0.076^{f}	0.038	1.25^{b-e}	0.269^{a-d}	2.06^{b-d}
OAK60	58.5^{ef}	2.23^{bc}	0.366^{e-g}	5.60^{a-d}	0.115^{cd}	0.032	1.29^{b-d}	0.247^{b-e}	2.11^{bc}
OAK80	59.9^{bc}	2.41^{ab}	0.400^{a-c}	5.44^{b-d}	0.148^{bc}	0.039	0.906^{ef}	0.193^{ef}	1.56^{de}
SED[4]	0.692	0.155	0.160	0.233	0.0210	0.0047	0.203	0.0349	0.278
P-value	<0.001	0.053	<0.001	0.099	<0.001	0.288	0.006	<0.001	<0.001
Contrasts[5]									
QUE	LQ	(L)	Q	(L)	L		L	L	L

GRA	LQ	(L)		(L)		L	Q	LQ
CHE	LQ	(L)	Q		LQ	L	LQ	L
OAK	LQC				Q	LQC	Q	LQC

[1]The incubated substrate was a TMR (forage:concentrate ratio 50:50) supplemented with 20 g of sunflower oil/kg diet DM.

[2]Coelutes with *trans*-7 *cis*-9 18:2 and *trans*-8 *cis*-10 18:2.

[3]PUFA, polyunsaturated fatty acids.

[4]SED=standard error of the difference.

[5]For each tannin, significance ($P<0.05$) of linear (L), quadratic (Q) and cubic (C) responses to tannin addition. Trends towards significance ($P<0.10$) are reported in parentheses.

[a-h]Within a column, different superscripts indicate significant differences.

Table 3

Effect of incremental levels of different tannin extracts (quebracho, QUE; grape, GRA; chestnut, CHE; oak, OAK) on odd- and branched-chain and oxo- fatty acid concentration (g/100 g of total fatty acids) after 12-h *in vitro* incubation with rumen inoculum from sheep[1].

	14:0 *iso*	15:0 *iso*	15:0 *anteiso*	15:0	17:0	BCFA[2]	OBCFA[3]	10-oxo-18:0	13-oxo-18:0
Control	0.068^{e}	0.261	0.431^{f}	1.06^{c-e}	0.955^{a}	2.34^{ef}	4.84^{de}	0.143^{b-d}	0.190^{d-f}
QUE20	0.085^{a-d}	0.305	0.489^{b-f}	1.13^{a-c}	0.851^{i}	2.63^{b-d}	5.13^{b-d}	0.101^{de}	0.164^{fg}
QUE40	0.093^{a-c}	0.320	0.519^{a-d}	1.18^{a}	0.862^{hi}	2.71^{ab}	5.24^{a-c}	0.103^{de}	0.166^{e-g}
QUE60	0.084^{a-d}	0.273	0.491^{b-f}	1.07^{c-e}	0.881^{fg}	2.59^{b-f}	5.06^{b-e}	0.096^{e}	0.134^{g}
QUE80	0.071^{de}	0.251	0.436^{ef}	1.04^{e}	0.947^{ab}	2.32^{f}	4.79^{e}	0.112^{de}	0.188^{d-f}
GRA20	0.077^{de}	0.309	0.535^{a-c}	1.09^{b-e}	0.884^{fg}	2.73^{ab}	5.28^{ab}	0.143^{b-d}	0.203^{c-e}
GRA40	0.075^{de}	0.287	0.502^{a-f}	1.07^{c-e}	0.878^{f-h}	2.61^{b-e}	5.09^{b-e}	0.194^{a}	0.222^{cd}
GRA60	0.075^{de}	0.289	0.528^{a-d}	1.07^{c-e}	0.890^{ef}	2.62^{b-e}	5.12^{b-d}	0.189^{a}	0.231^{bc}
GRA80	0.079^{c-e}	0.289	0.540^{a-c}	1.09^{b-e}	0.922^{cd}	2.64^{bc}	5.16^{b-d}	0.175^{ab}	0.231^{bc}
CHE20	0.095^{ab}	0.316	0.521^{a-d}	1.17^{a}	0.871^{gh}	2.68^{bc}	5.21^{a-c}	0.134^{b-e}	0.221^{cd}
CHE40	0.096^{ab}	0.318	0.513^{a-e}	1.16^{ab}	0.876^{f-h}	2.64^{bc}	5.19^{a-c}	0.123^{c-e}	0.262^{ab}
CHE60	0.098^{ab}	0.312	0.550^{ab}	1.11^{a-e}	0.894^{ef}	2.77^{ab}	5.29^{ab}	0.104^{de}	0.147^{g}
CHE80	0.074^{de}	0.255	0.455^{d-f}	1.06^{de}	0.934^{bc}	2.36^{d-f}	4.84^{de}	0.137^{b-e}	0.200^{c-f}
OAK20	0.099^{a}	0.352	0.578^{a}	1.13^{a-d}	0.876^{f-h}	2.96^{a}	5.49^{a}	0.172^{a-c}	0.294^{a}
OAK40	0.084^{b-d}	0.310	0.505^{a-f}	1.07^{c-e}	0.882^{fg}	2.69^{a-c}	5.21^{a-c}	0.178^{ab}	0.272^{a}
OAK60	0.077^{de}	0.304	0.519^{a-d}	1.07^{c-e}	0.907^{de}	2.67^{bc}	5.19^{a-c}	0.160^{a-c}	0.199^{c-f}
OAK80	0.074^{de}	0.264	0.470^{c-f}	1.09^{c-e}	0.957^{a}	2.41^{c-f}	4.94^{c-e}	0.144^{b-d}	0.200^{c-f}
SED[4]	0.0073	0.0336	0.0379	0.0372	0.0091	0.137	0.159	0.0243	0.0158
P-value	0.004	0.232	0.027	0.007	<0.001	0.003	0.010	<0.001	<0.001
Contrasts[5]									
QUE	LQ			Q	LQC			L	L

GRA				LQ	Q			
CHE	LQ	L	Q	LQC	L	L	L	QC
OAK	LQ	QC		LQ	QC	Q		Q

[1]The incubated substrate was a TMR (forage:concentrate ratio 50:50) supplemented with 20 g of sunflower oil/kg diet DM.

[2]BCFA, branched-chain fatty acids.

[3]OBCFA, odd- and branched-chain fatty acids.

[4]SED=standard error of the difference.

[5]For each tannin, significance of linear (L), quadratic (Q) and cubic (C) responses to tannin addition.

[a-i]Within a column, different superscripts indicate significant differences.

Table 4

Effect of the addition of an oak tannin extract (20 g/kg diet DM) on rumen fermentation parameters after *in vitro* incubation with rumen inoculum from sheep[1].

Item[2]	Control	OAK20	SED[3]	P-value
DMD_{72} (g/g)	0.703	0.679	0.0301	0.464
A (mL/g OM)	381	381	10.7	0.979
c (/h)	0.068	0.071	0.0016	0.176
AFR	18.7	19.5	0.355	0.115
ED (g/g)	0.435	0.426	0.0209	0.690
*iv*TSD (g/g)	0.809	0.802	0.0195	0.752
pH	6.44	6.46	0.0240	0.526
Ammonia (mg/L)	709	654	101	0.618
Total VFA (mmol/L)	79.2	71.5	4.72	0.176
Molar proportions (mol/mol)				
Acetate	0.613	0.602	0.0225	0.641
Propionate	0.177	0.182	0.0117	0.664
Butyrate	0.157	0.162	0.0095	0.665
Others[4]	0.053	0.054	0.0029	0.671
Acetate:propionate ratio	3.52	3.35	0.338	0.635

[1]The incubated substrate was a TMR (forage:concentrate ratio 50:50) supplemented with 20 g of sunflower oil/kg diet DM.

[2]*A* = cumulative gas production; AFR = average fermentation rate; *c* = fractional fermentation rate; DMD_{72} = DM disappearance after 72 h of incubation; ED = extent of degradation in the rumen; *iv*TSD = *in vitro* true substrate digestibility; VFA = volatile fatty acids.

[3]SED=standard error of the difference.

[4]Calculated as the sum of isobutyrate, isovalerate and valerate.

Table 5

Effect of the addition of an oak tannin extract (20 g/kg diet DM) on the diversity indices of the bacterial community, and on the relative frequencies [expressed as $\log_{10}$ (n + c) of the percentage over the total peak area, with original values in parentheses] of some terminal restriction fragments (T-RF) after 24-h *in vitro* incubation with rumen inoculum from sheep[1].

		Control	OAK20	SED[2]	P-value
Diversity indices					
HhaI	Richness	64.3	63.0	2.67	0.643
	Shannon index	3.58	3.59	0.0658	0.933
MspI	Richness	95.0	95.3	4.70	0.947
	Shannon index	4.11	4.15	0.0509	0.472
HaeIII	Richness	56.0	57.3	3.67	0.735
	Shannon index	3.61	3.62	0.0732	0.940
T-RF frequencies					
Clostridia[3]	750 bp (*HhaI*)	-0.064 (0.864)	0.099 (1.28)	0.0611	0.056
	95 bp (*MspI*)	0.836 (7.06)	0.836 (7.42)	0.0908	0.783
Lachnospiraceae[3]	65 bp (*HhaI*)	0.895 (7.87)	0.946 (8.82)	0.0138	0.022
	293 bp (*MspI*)	0.077 (1.20)	0.176 (1.50)	0.0247	0.016
	277 bp (*HaeIII*)	-0.056 (0.227)	0.291 (1.28)	0.109	0.034

[1]The incubated substrate was a TMR (forage:concentrate ratio 50:50) supplemented with 20 g of sunflower oil/kg diet DM.

[2]SED=standard error of the difference.

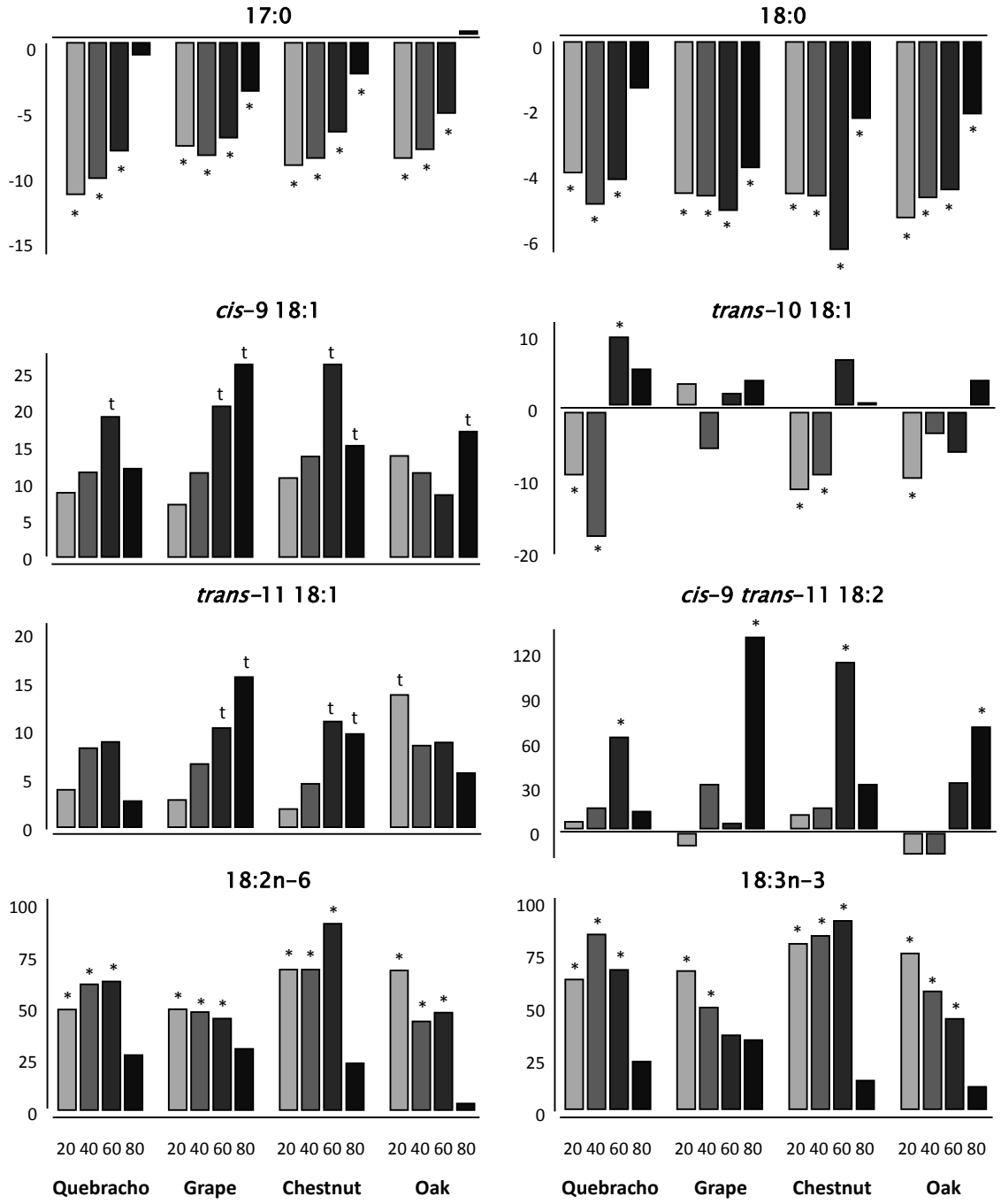
[3]Putative taxonomic identification.

FIGURE CAPTIONS

Figure 1. Effect of incremental levels of different tannin extracts (quebracho, grape, chestnut and oak) on the percentage of variation with respect to the control (*i.e.*, without tannins) of 17:0 (SEM=0.801), 18:0 (SEM=0.317), *cis*-9 18:1 (SEM=1.45), *trans*-10 18:1 (SEM=1.82), *trans*-11 18:1 (SEM=0.987), *cis*-9 *trans*-11 18:2 (SEM=10.5), 18:2n-6 (SEM=5.32), and 18:3n-3 (SEM=6.09) content after 12-h *in vitro* incubation with rumen inoculum from sheep.

Differences (*: $P<0.05$; t: $P<0.10$) compared with the control. SEM=standard error of the mean.

FIGURE 1


17:0
18:0
cis-9 18:1
trans-10 18:1
trans-11 18:1
cis-9 trans-11 18:2
18:2n-6
18:3n-3
20 40 60 80
Quebracho
Grape
Chestnut
Oak